

Direct Numerical Test of the Statistical Mechanical Theory of Hydrophobic Interactions

M. I. Chaudhari,^{1, a)} S. Holleran,^{1, b)} H. S. Ashbaugh,^{1, c)} and L. R. Pratt^{1, d)}

Department of Chemical and Biomolecular Engineering, Tulane University, New Orleans, LA 70118

This work tests the statistical mechanical theory of hydrophobic interactions, isolates consequences of excluded volume interactions, and obtains B_2 for those purposes. Cavity methods that are particularly appropriate for study of hydrophobic interactions between atomic-size hard spheres in liquid water are developed and applied to test aspects of the Pratt-Chandler (PC) theory that have not been tested. Contact hydrophobic interactions between Ar-size hard-spheres in water are significantly more attractive than predicted by the PC theory. The corresponding results for the osmotic second virial coefficient are attractive ($B_2 < 0$), and more attractive with increasing temperature ($\Delta B_2/\Delta T < 0$) in the temperature range $300 \text{ K} \leq T \leq 360 \text{ K}$. This information has not been available previously, but is essential for development of the molecular-scale statistical mechanical theory of hydrophobic interactions, particularly for better definition of the role of attractive intermolecular interactions associated with the solutes.

I. INTRODUCTION

Hydrophobic interactions are universally acknowledged as fundamental contributions to the stability of folded or aggregated biomolecular structures in water. But hydrophobic interactions are also expected to become more favorable with increasing temperature for physiological temperatures. On this basis, hydrophobic interactions are principally entropic interactions.

Hydrophobic interactions can then be described as favorable for aggregation *and endothermic* at moderate temperatures. The osmotic second virial coefficient

$$B_2 = \lim_{R \rightarrow \infty} \left\{ -2\pi \int_0^R [g_{AA}(r) - 1] r^2 dr \right\} \equiv \lim_{R \rightarrow \infty} B_2[R] \quad (1)$$

is the solution thermodynamic metric for assessment of attractive and repulsive character of hydrophobic interactions between an AA solute pair. Here $g_{AA}(r)$ is the usual radial distribution function of AA pairs at infinite dilution. Positive values of B_2 raise the osmotic pressure, and indicate preponderance of repulsive effects. Negative values of B_2 lower the osmotic pressure and characterize interactions that are attractive on balance.

The entropic aspect of hydrophobic interactions requires statistical thermodynamics for explanation. Since the molecular theory of hydrophobic interactions thus requires specified intermolecular interactions and defensible statistical mechanics, the theory of hydrophobic interactions has been intermittent and only partially successful. The approximate Pratt-Chandler (PC) theory¹ was the first prediction of molecular-scale $g_{AA}(r)$ for inert

atom solutes in water and thus the first molecular-scale prediction of B_2 . Though the PC theory predicted attractive and repulsive features in unprecedented detail, it did not straightforwardly conform to the expectation that hydrophobic interactions as expressed by B_2 should be *attractive and endothermic*. The PC theory was immediately controversial.^{2,3} Experiments for benzene, and perfluorobenzene^{4,5} disagreed with the PC theory for atomic-size hard-sphere solutes. Explanations for the discrepancy were suggested⁶⁻⁸ but the underlying controversy has persisted.

One challenge for addressing this controversy is that the integrated quantity B_2 , and particularly the $R \rightarrow \infty$ limit, is a subtle target for molecular simulation calculations. Another challenge is that this problem requires analysis of temperature (T) dependences in a limited T range.

But further challenges remain, and a summary of the substantial efforts to resolve this issue was given recently.¹¹ The broad conclusion is that simulation tests of the theory have differed enough in details that a conclusive test of the PC theory for hydrophobic interactions has been elusive. For example, B_2 's for the specific cases of Ar and CH₄ solutes have been estimated¹² to be remarkably small, due evidently to substantial cancellation between repulsive and attractive force effects. In the same context,¹² B_2 for Kr(aq) has been estimated to be repulsive (positive). Clearly, attractive and repulsive interactions can play conflicting roles with the consequence that merely realistic simulation of a case of interest, *e.g.*, CH₄, might not provide helpful physical conclusions. Indeed, the case of atomic-size hard-sphere solutes has not been treated specifically because hard-sphere models are inconvenient within common molecular dynamics tools.

As another example, solute non-pairwise interactions associated with polarizabilities were of simulation interest for a time.¹³ Those interests have waned inconclusively,^{13,14} undoubtedly because of a lack of a molecular theory that could sharpen the questions and make the answers more permanent. Such differences are indeed broadly expected to be details, but in this context

^{a)}Electronic mail: chaudhari.84@gmail.com

^{b)}Electronic mail: shollera@tulane.edu

^{c)}Electronic mail: hanka@tulane.edu

^{d)}Electronic mail: lpratt@tulane.edu

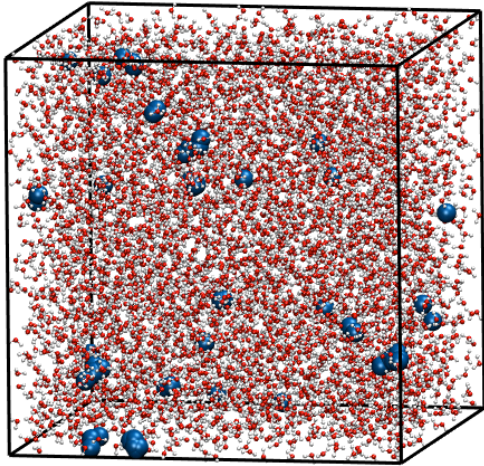


FIG. 1. A configuration of 5×10^3 water molecules (red and silver) together with the inclusions (blue spheres) identified by $n_t = 2 \times 10^5$ trial placements of a hard sphere with distance of closest approach to an oxygen atom of 0.31 nm. This size corresponds approximately to an Ar solute for which the van der Waals length parameter σ_A is about 0.34 nm,⁹ thus adopting 0.31 nm - 0.17 nm = 0.14 nm as a van der Waals contact radius of the water oxygen atom. Hard sphere solutes of this size are about maximal for water-oxygen contact density.¹⁰

those details have been features of controversy.

In parallel with simulation efforts, the foundation of the molecular theory of hydrophobic effects has undergone a surprising renovation.^{10,13} The renovated theory directly exploits molecular simulation data and substantially amends the PC theory, but gives unexpected support for some of the conceptual ingredients of that theory.¹³ For example, the concern that the PC theory neglected molecular orientational structure of liquid water is now generally recognized as not well founded. The same can be said about the scaled-particle approaches applicable to the present problem.^{10,15} The renovation of the molecular theory is mostly due to aggressive extension of the scaled-particle-theory concepts. Indeed, the success of the scaled-particle theory extensions^{10,16} relieves the most serious objections to the PC theory which can then be seen as merely an approximate theory of liquid solutions. Thus applied to molecular-scale problems, the PC theory has about the same conceptual status as the distinct minimal scaled-particle theories. Of course, the PC theory (and the information theory approach¹⁷ which is a hybrid) addresses hydrophobic interactions which have not been addressed by scaled-particle approaches.

Here we address the issue of B_2 from the renovated point-of-view. We find that B_2 for atomic-size hard spheres in water is attractive ($B_2 < 0$), and more attractive with increasing temperature ($\Delta B_2 / \Delta T < 0$).

II. THEORY

We seek the *cavity correlation function*

$$y_{AA}(r) = \exp[u_{AA}(r)/k_B T] g_{AA}(r) \quad (2)$$

for atomic-size hard spheres on the basis of the potential distribution theorem (or *test particle*) approach^{18,19}

$$y_{AA}(1, 2) = \frac{\langle e^{-\beta \Delta U_{AA}^{(2)}} | 1, 2 \rangle_0}{\langle e^{-\beta \Delta U_A^{(1)}} \rangle_0 \langle e^{-\beta \Delta U_A^{(1)}} \rangle_0} \quad (3)$$

This formula is cast for evaluation on the basis of trial placements of hard-spheres at specific points, here the two points (1, 2). Of course, we expect $y_{AA}(1, 2) = y_{AA}(r)$ to depend only on the magnitude r of the displacement between positions 1 and 2. The notation $\langle \dots \rangle_0$ indicates the average over the configurations of water without the solutes present. $\Delta U_A^{(1)} = U(N+1) - U(N) - U(1)$ is the binding energy for insertion of an A atom, and for the hard sphere case considered here is either zero (no overlap with a water oxygen atom) or positive infinity. Thus $e^{-\beta \Delta U_A^{(1)}}$ is an indicator function for permissibility of an insertion at a point considered. We will treat the case that $\Delta U_{AA}^{(2)}$ for two trial placements is additive, $\Delta U_A^{(1)} + \Delta U_A^{(1)}$ for the AA atoms considered.

We rearrange this formula to make the numerical estimation transparent, and this rearrangement overlaps a recent discussion of the Kirkwood-Salzburg theory.¹⁸ Note that the denominator factors of Eq. (3), being averages of indicator functions, are probabilities. Following the primitive understanding of conditional probabilities $p(A|B) = p(AB)/p(B)$, we use one of those denominator probabilities to introduce the expectation *conditional* on permissibility of that initial insertion. Taking the position of that first insertion to be the origin $\mathbf{0}$ we write

$$y_{AA}(r) = \frac{\langle e^{-\beta \Delta U_A^{(1)}} | \mathbf{r} \rangle_0}{\langle e^{-\beta \Delta U_A^{(1)}} \rangle_0}, \quad (4)$$

where \mathbf{r} is the position of a trial placement relative to a permissible insertion. The average indicated in the numerator is conditional on the permissible placement at $\mathbf{0}$, though we do not set-up a further notation for that. Eq. (4) expresses the well-known zero-separation theorem²⁰⁻²²

$$y_{AA}(0) = \frac{1}{\langle e^{-\beta \Delta U_A^{(1)}} \rangle_0}, \quad (5)$$

since the numerator is one (1) under the condition of a permissible insertion at $\mathbf{0}$.

To estimate the ratio (Eq. (4)) we exploit *many* (n_t) trial placements into the system volume V , for each one of n_c configurations, c (Fig. 1). Those trial points will have the density n_t/V and are statistically uniform. Out of the n_t trial points, a smaller number $n_s(c)$ are permissible

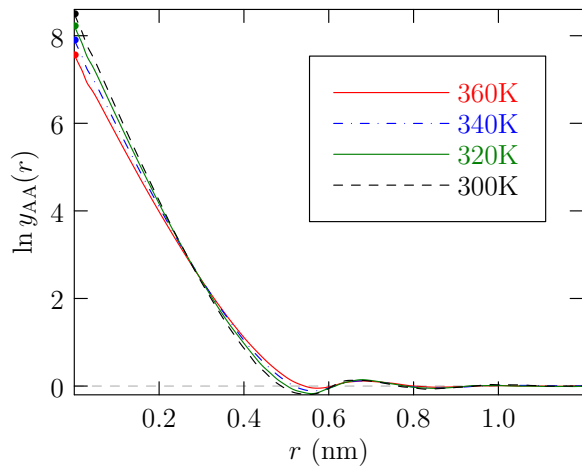


FIG. 2. Cavity correlation functions for hard-sphere solutes in liquid water at constant pressure $p = 1$ atm, and four different temperatures. The spheres have van der Waals radius of 0.17 nm and distance-of-closest-approach to a water oxygen atom of 0.31 nm. The dots on the left vertical axis are values calculated independently on the basis of the zero-separation theorem.

placements, and we estimate the denominator of Eq. (4) with $\sum_c n_s(c) / (n_c n_t) = \bar{n}_s / n_t$.

We expect $(n_t - 1) \Delta V / V$ of those trial placements to land in a volume ΔV which is a thin shell of radius $r > 0$ surrounding a permissible insertion. Let's denote by $\Delta n_s(r; c)$ the number of permissible placements obtained in the shell for configuration c . We estimate the numerator of Eq. (4) as

$$\frac{\sum_c \Delta n_s(r; c)}{(n_t - 1) n_c \Delta V / V} = \frac{\Delta \bar{n}_s(r)}{(n_t - 1) \Delta V / V}. \quad (6)$$

Combining these results, we have the estimate

$$\left(\frac{\bar{n}_s}{V} \right) y_{AA}(r) \Delta V = \Delta \bar{n}_s(r), \quad (7)$$

when $n_t \rightarrow \infty$. This is the same formula as if the permissible insertions were actual particles though that is not the case here.

The formula Eq. (7) is operationally consistent with the zero-separation theorem Eq. (5) according to the following argument. Consider a small volume element surrounding the position $\mathbf{r} = \mathbf{0}$ that is known to be a permissible placement. We expect that all trial placements in this region should be permissible so $\Delta \bar{n}_s(\mathbf{0}) \approx n_t \Delta V / V$ on the right-side of Eq. (7). Therefore, $y_{AA}(0) \approx n_t / \bar{n}_s$ which is the operational content of Eq. (5).

III. RESULTS AND DISCUSSION

Using standard methods (detailed below), this approach was implemented for the case that the hard-sphere distance-of-closest-approach to an oxygen atom

was 0.31 nm, corresponding approximately to the case of an Ar solute. Larger solutes would make the present calculations prohibitively difficult. The results for $\ln y_{AA}(r)$ (Fig. 2) operationally satisfy the zero-separation theorem, show strong hydrophobic attraction as short-distances, and solvent-separated hydrophobic attraction qualitatively consistent with the PC theory.

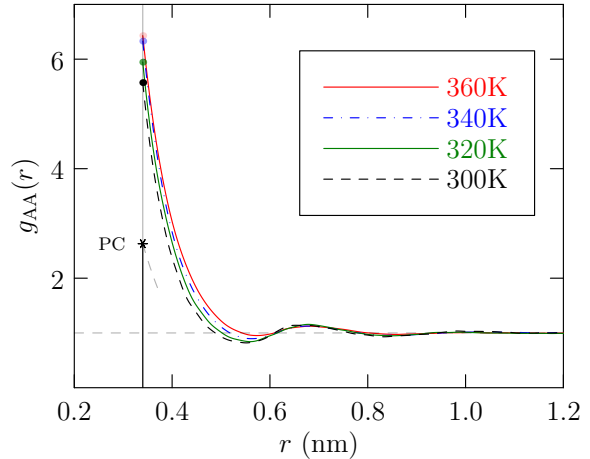


FIG. 3. Radial distribution functions for hard-sphere solutes in liquid water at constant pressure $p = 1$ atm, and four different temperatures. The spheres have van der Waals radius of 0.17 nm and distance-of-closest-approach to a water oxygen atom of 0.31 nm. The prediction of the PC theory¹ at $T = 300$ K for the contact value, $g_{AA}(r = 0.34 \text{ nm})$, is shown by the star. The contact values determined here are consistent with the information theory model¹⁷ result published previously, and more than twice larger than the predictions of the PC theory. These contact values are higher for higher temperatures, indicating stronger hydrophobic contact attractions at higher temperatures.

The radial distribution function $g_{AA}(r) = y_{AA}(r)$ for $r \geq 2 \times 0.17 \text{ nm} = 0.34 \text{ nm}$ (and zero otherwise, Fig. 3), scrutinizes these properties more closely. The contact values, $g_{AA}(r = 0.34 \text{ nm})$, determined here are consistent with the information theory model result published previously,¹⁷ and more than twice larger than the predictions of the PC theory. The contact values are higher for higher temperatures, indicating stronger hydrophobic contact attractions at higher temperatures. The contact values of the PC theory also increase with increasing T ¹ but those increases are small, and the PC contact values are sufficiently different from the numerical values found here that the small increases are not interesting.

It is commonplace for simulation calculations to determine the temperature dependences of the AA potentials of mean force $w_{AA}(r) \equiv -k_B T \ln g_{AA}(r)$. Some examples are²³⁻³⁰. We note below that the recent work of that type supports our conclusions here (Fig. 3). But that helpful recent work also addresses our present problems only indirectly because it does not test the statistical mechanical theory, nor isolate aspects of interactions of different physical type, nor obtain B_2 for those purposes.

Based on the experience, the measurable osmotic second virial coefficients are more subtle, and despite interesting suggestions,³¹ $w_{AA}(r)$'s have not been measured for molecular-scale hydrophobic solutes.

The B_2 integrals (Fig. 4) provide the solution thermodynamic assessment of these distribution functions. The suggested B_2 values are decidedly attractive (negative) for $T = 300$ K and become more attractive at higher temperatures. The biggest negative contribution is associated with contact hydrophobic attractions. Solvent-separated hydrophobic attractions near $R \approx 0.7$ nm are distinct but smaller than contact hydrophobic interactions.

The significance of the solvent-separated hydrophobic interactions has been much discussed following the ground-breaking work of Pangali, *et al.*^{32,33}. Those simulations treated Lennard-Jones (LJ) model solutes somewhat similar to Kr or Xe solutes. Simulation results (with LJ attractions) were compared with PC theory (not treating LJ attractions though modified for continuous repulsive interactions). In view of the introductory discussion, further analysis of the role of attractive interactions should be helpful. But additionally, since the simulation did not determine the additive constant to $w_{AA}(r)$, the comparison proceeded after matching the two results at their minimum values, a convenient choice that has been sometimes followed.^{14,34} This comparison can give the impression that the non-matched solvent-separated hydrophobic interactions are unusually variable or significant. If, for the purposes of comparison, the $w_{AA}(r)$ were matched at solvent-separated radii, then the Pangali, *et al.* results show stronger contact hydrophobic attractions than does the PC theory, qualitatively in agreement with the present work. Recent molecular dynamics simulations for Xe(aq) or CH₄(aq) pairs^{28–30} agree qualitatively with the present results and thus support this conclusion. Still the role of longer-ranged attractive interactions remains to be studied.

Inclusion of longer-ranged attractive interactions, *i.e.*, London dispersion interactions, can change these B_2 values and trends depending on the balance of solute-solute and solute-water interactions.^{8,11} Conclusive information for the case that more general interactions are *absent*, as with this work, is crucial to justifying further analysis of more general interactions. The LMF theory³⁵ is one promising suggestion for how to proceed with inclusion of longer-ranged interactions, has commonalities with earlier intuitive proposals,^{8,11} and deserves further development.

The temperature dependence observed here has generally been considered counter-intuitive, and is sometimes referred to as an *inverse* temperature dependence. An explanation why this behavior might be considered counter-intuitive is the following³⁶: hydrophobic association is typically rationalized as clumping of inert solution inclusions due to specific structuring of their hydration shells. It might be guessed that the specific structuring should be more significant at lower temperatures, so per-

haps the hydrophobic association should be stronger at lower temperatures, perhaps even more important yet in super-cooled water.

Hydration-shell structuring surely is an important factor in hydrophobic interactions. What this argument does not address is the distinctive equation of state of liquid water. Some well recognized peculiarities occur at higher-than-physiological temperatures; for example the compressibility minimum occurs at 46 C, under these low pressure conditions. The eventual statistical mechanical explanation³⁷ of the similarly counter-intuitive *entropy convergence* hydrophobic phenomenon (at $T \approx 130$ C) depended firstly on proper involvement of the actual equation of state of liquid water.¹⁰ Indeed the calculations here model that specific equation of state also.

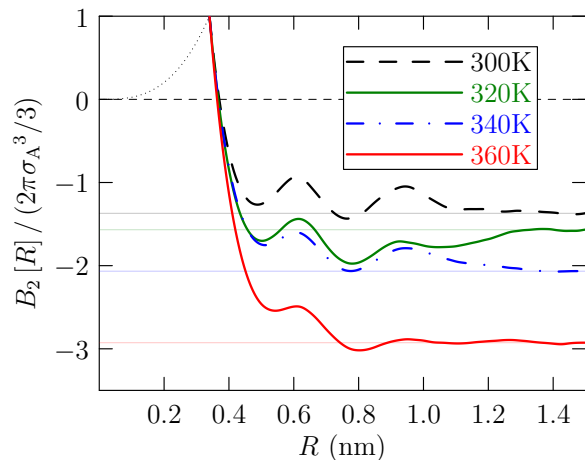


FIG. 4. Running integral $B_2[R]$ (Eq. (1)) for assessment of the $R \rightarrow \infty$ value. The dotted curve for $R \leq 0.34$ nm is the positive contribution from the hard-core of $g_{AA}(r)$ that is common to all here. The suggested $R \rightarrow \infty$ values are decidedly negative (attractive) and become more attractive at higher temperatures. The biggest negative contribution is associated with contact hydrophobic attractions. Solvent-separated hydrophobic attractions near $R \approx 0.7$ nm are distinct but smaller. The predictions of the PC theory for B_2 in these circumstances are repulsive (positive).

IV. CONCLUSIONS

This work tests the statistical mechanical theory of hydrophobic interactions, isolates consequences of excluded volume interactions, and obtains B_2 for those purposes. Contact hydrophobic interactions between Ar-size hard-spheres in water are significantly more attractive than predicted by the Pratt-Chandler theory. The corresponding B_2 results for atomic-size hard spheres in water are attractive ($B_2 < 0$), and more attractive with increasing temperature ($\Delta B_2 / \Delta T < 0$) in the temperature range $300 \text{ K} \leq T \leq 360 \text{ K}$. This information is essential for further development of the molecular-scale statistical mechanical theory of hydrophobic interactions.

V. METHODS

The GROMACS³⁸ package and the SPC/E³⁹ model was used to simulate liquid water. This simulation adopted the isothermal-isobaric ensemble at four different temperatures ($T = 300$ K, 320 K, 340 K and 360 K). A Nose-Hoover thermostat maintained the temperature and a Parinello-Rahman barostat was used to establish the pressure at 1 atm. Bonds involving hydrogen atoms were constrained by the LINCS algorithm. Conventional periodic boundary conditions and particle mesh Ewald, with a real-space cut-off at 1 nm, was used to treat long-range interactions. Simulation cells containing 5×10^3 randomly placed water molecules were created utilizing PACKMOL⁴⁰ to match the experimental density approximately. After 1×10^4 steps of energy minimization and 2 ns of density equilibration, trajectories of 20 ns (sampled 1/ps) were obtained at each temperature. Each simulation frame was analyzed for cavities based on $n_t = 2 \times 10^5$ trial placements with a distance of closest approach to an oxygen atom of 0.31 nm. Successful placements can be considered as hard spheres of radius 0.17 nm corresponding approximately an Ar atom. The cavity analysis is about an order-of-magnitude more computational effort than the generation of the molecular dynamics trajectories.

Considering the integrand of Eq. (1), $g_{AA}(r) \sim 1$ in the thermodynamic limit. For a fixed particle numbers in a finite system, that subtracted uncorrelated feature is less than 1 and the correction is $O(V^{-1})$ ⁴¹, vanishing in the thermodynamic limit. In the present work, we do not have fixed numbers of A particles.

- ¹L. R. Pratt and D. Chandler, J. Chem. Phys. **67**, 3683 (1977).
- ²D. Y. C. Chan, D. J. Mitchell, B. W. Ninham, and B. A. Pailthorpe, in *Recent Advances, Water: A Comprehensive Treatise*, Vol. 6, edited by F. Franks (Plenum, New York, 1979) pp. 239–278.
- ³P. J. Rossky and H. L. Friedman, J. Phys. Chem **84**, 587 (1980).
- ⁴E. E. Tucker and S. D. Christian, J. Phys. Chem **83**, 426 (1979).
- ⁵P. J. Bernal, S. D. Christian, and E. E. Tucker, J. Soln. Chem. **15**, 947 (1986).
- ⁶L. R. Pratt and D. Chandler, J. Soln. Chem. **9**, 1 (1980).
- ⁷L. R. Pratt and D. Chandler, J. Chem. Phys. **73**, 3430 (1980).
- ⁸L. R. Pratt and D. Chandler, J. Chem. Phys. **73**, 3434 (1980).
- ⁹T. L. Hill, *An Introduction to Statistical Thermodynamics* (Addison-Wesley, Reading MA USA, 1960).
- ¹⁰H. S. Ashbaugh and L. R. Pratt, Rev. Mod. Phys. **78**, 159 (2006).
- ¹¹D. Asthagiri, S. Merchant, and L. R. Pratt, J. Chem. Phys. **128**, 244512 (2008).

- ¹²K. Watanabe and H. C. Andersen, J. Phys. Chem. **90**, 795 (1986).
- ¹³L. R. Pratt, Ann. Rev. Phys. Chem. **53**, 409 (2002).
- ¹⁴W. S. Young and C. L. Brooks, J. Chem. Phys. **106**, 9265 (1997).
- ¹⁵F. H. Stillinger, J. Soln. Chem. **2**, 141 (1973).
- ¹⁶H. S. Ashbaugh and L. R. Pratt, J. Phys. Chem. B **111**, 9330 (2007).
- ¹⁷G. Hummer, S. Garde, A. E. García, A. Pohorille, and L. R. Pratt, Proc. Natl. Acad. Sci. USA **93**, 8951 (1996).
- ¹⁸T. L. Beck, M. E. Paulaitis, and L. R. Pratt, *The Potential Distribution Theorem and Models of Molecular Solutions* (Cambridge University Press, 2006).
- ¹⁹D. M. Rogers, D. Jiao, L. R. Pratt, and S. B. Rempe, Ann. Rep. Comp. Chem. **8**, 71 (2012).
- ²⁰W. G. Hoover and J. C. Poirier, J. Chem. Phys. **37**, 1041 (1962).
- ²¹E. Meeron and A. J. F. Siegert, J. Chem. Phys. **48**, 3139 (1968).
- ²²J. A. Devore and J. E. Mayer, J. Chem. Phys. **70**, 1821 (1979).
- ²³D. E. Smith, L. Zhang, and A. D. J. Haymet, J. Am. Chem. Soc. **114**, 5875 (1992).
- ²⁴D. E. Smith, , and A. D. J. Haymet, J. Chem. Phys. **98**, 6445 (1993).
- ²⁵N. T. Skipper, Chem. Phys. Letts. **207**, 424 (1993).
- ²⁶S. Ludemann, H. Schreiber, R. Abseher, and O. Steinhauser, J. Chem. Phys. **104**, 286 (1996).
- ²⁷S. Ludemann, R. Abseher, H. Schreiber, and O. Steinhauser, J. Am. Chem. Soc **119**, 4206 (1997).
- ²⁸D. Paschek, J. Chem. Phys. **120**, 6674 (2004).
- ²⁹D. Paschek, J. Chem. Phys. **120**, 10605 (2004).
- ³⁰E. Sobolewski, M. Makowski, S. Oldziej, C. Czaplowski, A. Liwo, and H. A. Scheraga, Prot. Eng. Design and Selection **22**, 547 (2009).
- ³¹M. I. Chaudhari, L. R. Pratt, and M. E. Paulaitis, J. Chem. Phys. **133**, 231102 (2010).
- ³²C. Pangali, M. Rao, and B. J. Berne, J. Chem. Phys. **71**, 2975 (1979).
- ³³C. Pangali, M. Rao, and B. J. Berne, J. Chem. Phys. **71**, 2982 (1979).
- ³⁴M. I. Chaudhari and L. R. Pratt, in *OIL SPILL REMEDIATION: COLLOID CHEMISTRY-BASED PRINCIPLES AND SOLUTIONS*, edited by P. Somasundaran, R. Farinato, P. Patra, and K. D. Papadopoulos (John Wiley and Sons, Inc., 2012) see also: arXiv:1208.0349v2.
- ³⁵J. D. Weeks, Ann. Rev. Phys. Chem. **53**, 533 (2002).
- ³⁶F. H. Stillinger, Science **209**, 451 (1980).
- ³⁷S. Garde, G. Hummer, A. García, M. Paulaitis, and L. Pratt, Phys. Rev. Letts. **77**, 4966 (1996).
- ³⁸B. Hess, C. Kutzner, D. Van Der Spoel, and E. Lindahl, J. Chem. Theory Comp. **4**, 435 (2008).
- ³⁹H. J. C. Berendsen, J. R. Grigera, and T. P. Straatsma, J. Phys. Chem. **91**, 6269 (1987).
- ⁴⁰L. Martínez, R. Andrade, E. G. Birgin, and J. M. Martínez, J. Comp. Chem. **30**, 2157 (2009).
- ⁴¹J. L. Lebowitz and J. K. Percus, Phys. Rev. **122**, 1673 (1963).